

## Effect of the Inhibition of Protein Synthesis on the *Escherichia coli* Cell Envelope

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The consequences for cell envelope integrity of *Escherichia coli* K-12 of the inhibition of protein synthesis by a variety of means have been examined. Protein synthesis was blocked by the antibiotics chloramphenicol and streptomycin, by amino acid starvation of an amino acid auxotroph, and by inactivation of temperature-sensitive aminoacyl transfer ribonucleic acid synthetase and ribosomal mutations. Closely similar morphological and physiological effects were found irrespective of the means by which protein synthesis was blocked. Scanning electron microscopy revealed a spectrum of changes after protein inhibition, with granular material derived from cells and spheroplasts commonly seen. Streptomycin caused additional changes manifested in a collapsed appearance of treated cells. Measurements of the release of lipopolysaccharide from the cell surface, alterations in outer membrane penetrability, and lysis of lysozyme-ethylenediaminetetraacetic acid-treated cultures also showed that the various inhibitory treatments all had similar effects on cell envelope properties. The close correspondence between the effects seen with antibiotic-treated cultures and those in which protein synthesis inhibition was achieved by use of mutants indicates that the effects of chloramphenicol and streptomycin on the cell envelope are indirect consequences of ribosomal block, rather than due to multiple sites of action of the antibiotics.

There have been numerous reports of the morphological changes induced by the treatment of gram-negative bacteria with antibiotics that inhibit cell wall synthesis, but fewer have concerned themselves with the consequences of the changes in cultures treated with various antibiotics, including chloramphenicol and streptomycin, and Bergersen (3) examined stained preparations of chloramphenicol-treated cells. The effect of chloramphenicol on *Escherichia coli* was studied by Morgan et al. (20) by electron microscopy of thin sections, and more recently the scanning electron microscope (SEM) has been used by Klainer and Perkins (17) to observe the morphologies of organisms exposed to a number of antibiotics known to inhibit ribosomal function. Pulvertaft, Bergersen, and Klainer and Perkins all described aberrant forms of *E. coli* from antibiotic-treated cultures; these forms had enlarged cells, swollen cells resembling spheroplasts, filaments, branching forms, and other evidence of surface disruption.

Physiological and biochemical evidence for an effect of ribosome-active antibiotics on cellu-

lar morphology and surface integrity has also been presented. Allison et al. (2) showed by using an electronic particle counter that the size distribution of a culture of *E. coli* changed during chloramphenicol treatment, and Wyatt (31), using laser scattering techniques, has found changes in cell size distribution in a culture of *Pseudomonas aeruginosa* exposed to neomycin. Matzura and Broda (19) and Nor-mark and Westling (22) have shown that chloramphenicol causes a change in the permeability properties of the outer membrane, and it has been shown that chloramphenicol treatment results in the release from cells of *E. coli* and *Salmonella typhimurium* of a lipopolysaccharide (LPS)-phospholipid-protein complex derived from the outer membrane of the cell (7, 25). Similar changes in the outer membrane occur when protein synthesis is blocked by starving an auxotroph of a required amino acid (18, 19, 22, 25) or by the inactivation of a conditionally defective mutant component of the protein-synthesizing machinery (26).

### MATERIALS AND METHODS

**Organisms.** All strains used were derivatives of *E.*

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*coli* K-12, and their characteristics are described in Table 1.

**Media and culture methods.** The defined medium used was as described by Adelberg and Burns (1). Nutrient broth consisted of Oxoid nutrient broth no. 2 plus 0.3% yeast extract. Cultures were grown in 125-ml Erlenmeyer flasks equipped with side arms to allow the optical density to be followed on a Klett-Summerson colorimeter. The flasks were incubated in a New Brunswick gyratory water bath shaker.

In all experiments, cultures in the logarithmic phase of growth were diluted back to a Klett value of 20 (about  $10^8$  cells/ml) before the application of the inhibitory treatment (i.e., antibiotic addition, amino acid starvation, or temperature shift). Protein synthesis inhibition was in all cases virtually complete, as evidenced by the fact that the Klett value rose no more than 2 or 3 units over the course of a 3-h experiment. Starvation of auxotrophic strains was established by washing cells by centrifugation and resuspending them in the defined medium without arginine.

**SEM.** Cells to be examined by SEM were washed by centrifugation in 0.9% NaCl and fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Specimens were prepared for microscopy as described before (16) and examined in a Cambridge S4-10 SEM.

**Release of outer membrane material.** Outer membrane material released from cells was determined by measuring LPS by the incorporation of [ $^{14}$ C]galactose into acid-precipitable material by *galE* mutants according to the method of Rothfield and Pearlman-Kothencz (25). Cultures were grown in glycerol minimal medium to which [ $^{14}$ C]galactose ( $2 \times 10^{-5}$  M,  $11 \times 10^6$  counts per min per  $\mu$ mol) was added at zero time. Samples (3 ml) of the test culture were centrifuged at  $10,000 \times g$  for 15 min to deposit bacteria, and 1 ml of supernatant was removed into 1 ml of 10% cold trichloroacetic acid. Acid-precipitable material was then collected by membrane (Millipore Corp.) filtration and washing with 10 ml of 5% trichloroacetic acid. Radioactivity on the filters was determined by liquid scintillation counting. Values for the amount of LPS released were corrected for the optical density of the culture at time of sampling.

**Outer membrane permeability.** A measure of outer membrane permeability was obtained by finding the change in the susceptibility of cells to rifampin at a low concentration (5  $\mu$ g/ml), which kills cells at a rate limited by their permeability to the antibiotic. Reid and Speyer (24) showed that the barrier to the entry of rifampin lay in the outer membrane and that a maximal killing rate was only achieved at concentrations of 100  $\mu$ g/ml or more. To assess whether the susceptibility of organisms to the killing action of rifampin was altered by blocking of protein synthesis, the lethality of 100  $\mu$ g of rifampin per ml was also measured. After 3 h of incubation under the conditions specified in the text, rifampin was added to cultures to give the desired concentration. Twenty minutes later, samples were removed and the number of cells capable of forming colonies at 32 C was measured.

**Lysozyme-EDTA treatment.** The method of So-

TABLE 1. *Strains of E. coli K-12*<sup>a</sup>

Strain	Relevant mutation	Other markers	Derivation
JP5121	<i>pheS353</i>	<i>arg galE str<sup>r</sup></i>	This laboratory <sup>b</sup>
JP5128		<i>arg galE str<sup>r</sup></i>	TS <sup>+</sup> transducent of JP5121
JP5109	<i>argE3</i>	<i>his ilv trp galE str<sup>r</sup></i>	This laboratory
C1713	<i>str<sup>r</sup>s</i>	<i>met rel</i>	S.-S. Kang (14)
C1714		<i>met rel</i>	Mutant of C1713 (14)

<sup>a</sup> Numbers refer to allele numbers allotted to mutations in this laboratory.

<sup>b</sup> For descriptions of some characteristics of strains carrying *pheS353*, see references 26 and 27. In reference 26, *pheS353* was in error listed as *pheS352*.

kawa et al. (30) was followed. Cultures were centrifuged, and the cells were suspended in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.0) containing 40 mM sodium azide, 1 mM ethylenediaminetetraacetic acid (EDTA), and 20  $\mu$ g of lysozyme per ml at an absorbancy at 660 nm of approximately 0.5. The change in absorbancy at 660 nm at room temperature was followed with a Gilford 300 spectrophotometer.

## RESULTS

**SEM studies.** The morphologies of organisms subjected to treatments that block protein synthesis are shown in Fig. 2 through 6. Control cultures (Fig. 1) consisted almost exclusively of apparently normal rods; cells from cultures grown in nutrient broth or minimal medium were closely similar, and cells grown at 42 C resembled those grown at 32 C. Cultures subjected to chloramphenicol contained a range of cell forms: apart from normal cells and elongated (but otherwise normal) cells, many showed surface blebs and attached granular material (Fig. 2). Objects identified as spheroplasts, emerging generally at the midpoint of long cells that were presumably approaching division, are also seen (Fig. 3), and these resemble the spheroplasts induced by antibiotics inhibiting cell wall synthesis (12, 15, 21). The extent of spheroplasting and resultant lysis was not sufficient to cause a drop in the optical density of the culture. Organisms from a culture of strain JP5121 (*pheS353*) grown at 42 C, the temperature at which protein synthesis in *pheS353* strains is blocked due to inactivation of phenylalanyl transfer ribonucleic acid synthetase (26), is shown in Figure 4; granular material, as well as spheroplasts of various sizes, are seen. The largest spheroplasts we observed had a diameter of about 5  $\mu$ m and so were similar in size to those described by Nishino and Nakazawa (21) in penicillin-treated cells. Cells from a culture of an arginine auxotroph that had been deprived of arginine for 3 h included a

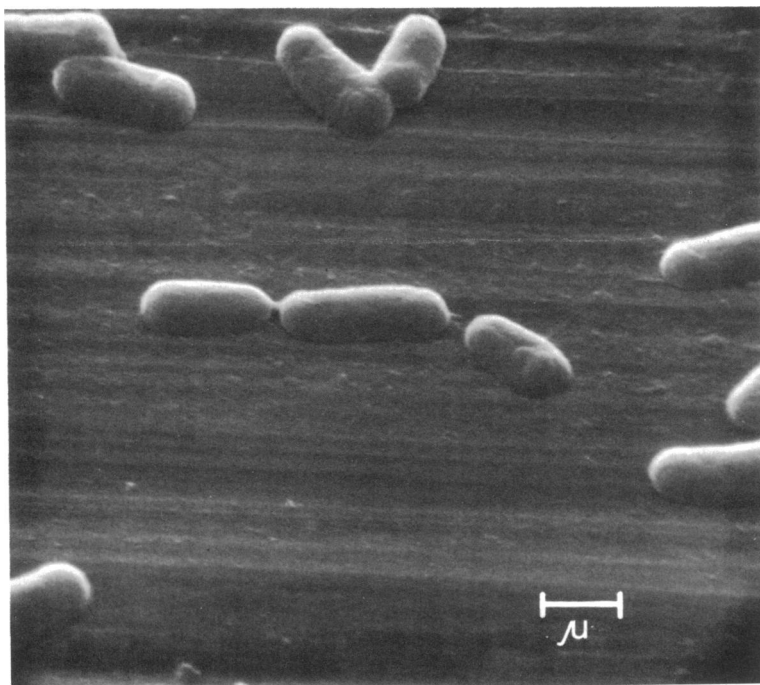


FIG. 1. JP5128 grown at 42 C.

range of aberrant forms like those induced by other inhibitory treatments.

Scanning electron photomicrographs of *E. coli* treated with streptomycin have not previously been reported, and Fig. 5 shows what happened to strain C1713 that had been exposed to 200  $\mu$ g of streptomycin per ml for 3 h. In addition to the effects seen with other means of inhibiting protein synthesis, nearly all the cells had a collapsed appearance, which agrees with the evidence of extensive membrane damage caused by streptomycin (8). Strain C1714 (*str<sup>ts</sup>*) is derived from C1713, and appears to possess a temperature-sensitive mutation in a ribosomal protein. Kang (14) has shown how this mutant responds to exposure to 42 C in a manner similar to C1713 treated with streptomycin, and Fig. 6 (top) illustrates the collapsed appearance of C1714 cells grown at 42 C and granular material previously demonstrated; spheroplasts were also seen.

The findings presented above essentially confirm and extend those reported previously (17) to show that very similar morphological changes are induced when protein synthesis is inhibited by any of a number of means. The nature of the granular matter is still unknown; it could consist of intracellular material released from burst spheroplasts or might alternatively be derived from the outer membrane, which is disrupted as a consequence of protein synthesis inhibition

(see below). The electron micrographs of Knox et al. (18) showed outer membrane blebbing off all over the cell surface, whereas the granular material we observed was typically conglomerated at a single point, perhaps as a consequence of the particular means of specimen preparation. There has been a previous report of outer membrane material detaching from the cell and forming up into coiled masses; this material may accumulate at one end of the cell (4), and certain physical treatments can cause ballooning of the outer membrane at individual sites on the cell surface (10, 29). Attempts to identify the granular matter as being derived from the outer membrane by investigating the attachment of phage T4 to it, as suggested by De Pamphilis (9), have been confounded by the close correspondence in size and appearance of T4 and granular matter viewed by SEM. Granular material is also observed in cultures treated with penicillin but, since penicillin causes outer membrane disruption (6; our unpublished observations) as well as its well-known spheroplasting activity, the results do not help identify it.

**Release of outer membrane material.** Knox et al. (18) showed some years ago that a lysine auxotroph of *E. coli*, when grown under lysine-limiting conditions, released into the growth medium a complex, derived from the outer membrane, that consisted of lipopolysaccha-

ride, phospholipid, and protein. Later workers (7, 25) extended these observations and found that limitation of amino acids other than lysine, as well as inhibition of protein synthesis by chloramphenicol, caused release of outer membrane, too. Control cells release very little LPS into the medium (about 2% of their total LPS synthesis), but as Fig. 7 indicates, there is a marked increase in the amount of LPS released in all cases when protein synthesis is blocked.

#### Changes in outer membrane permeability.

Previous reports have demonstrated changes in the susceptibility of cultures in which protein synthesis has been blocked by agents such as lysozyme, sodium deoxycholate, actinomycin D, or rifampin, all of which are normally restricted from entering the cell by the outer membrane (18, 19, 22, 26). Table 2 shows how inhibition of protein synthesis by chloramphenicol or the *pheS353* mutation increased the

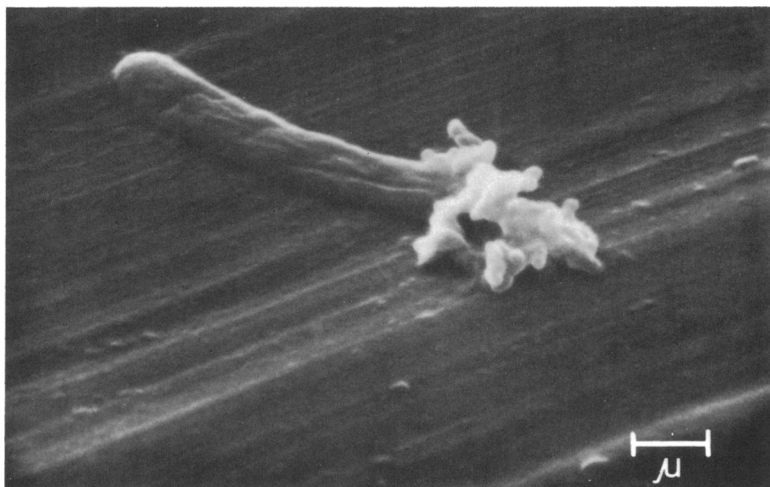


FIG. 2

FIG. 2 AND 3. *JP5128* treated with 200  $\mu$ g of chloramphenicol per ml for 3 h at 42 C.

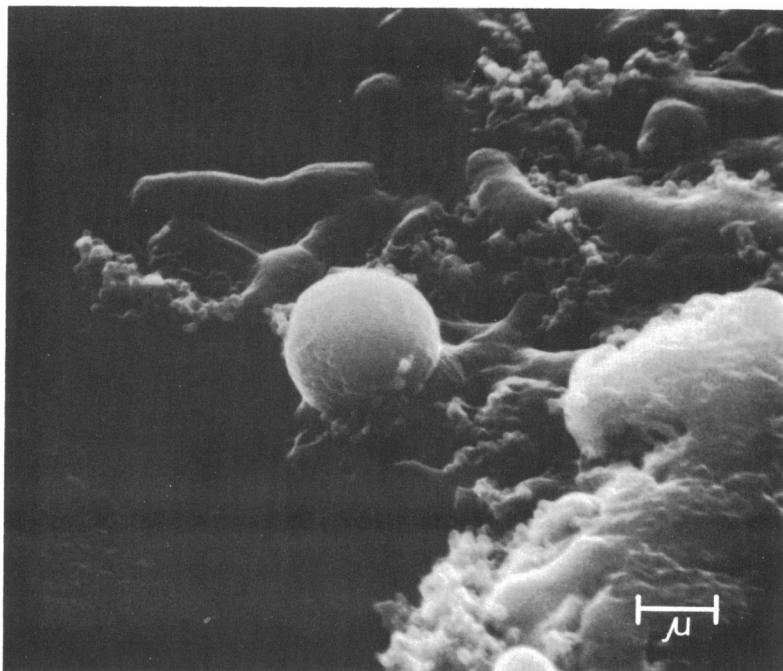


FIG. 3

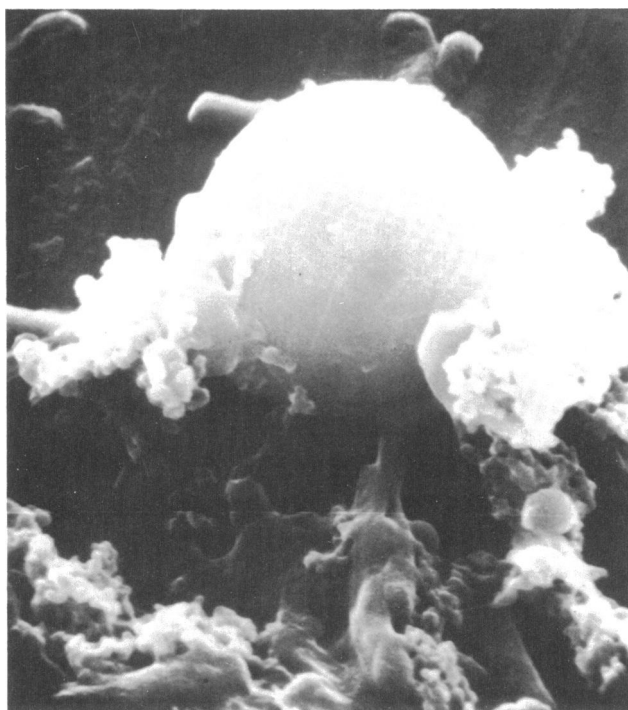


FIG. 4. JP5121 (*pheS353*) after 3 h at 42 C.

susceptibility of cells to a low concentration of rifampin, indicating that access of the antibiotic to the cytoplasm was enhanced. Inhibition of protein synthesis resulted in a decrease in the lethality of 100  $\mu$ g of rifampin per ml, and this decrease in fact partially masked the extent of the change in permeability to 5  $\mu$ g/ml. The amino acid auxotroph JP5109 also shows altered outer membrane permeability, measured by other techniques, when deprived of arginine at 42 C (A. F. Egan, personal communication).

**Cytoplasmic membrane changes.** Sokawa et al. (30) recently showed that cells from amino acid-starved cultures lysed more readily than unstarved controls when treated with lysozyme and EDTA, and took this as evidence of membrane alteration under starvation conditions. Figure 8 confirms this effect for amino acid-starved cells and shows the similar result obtained with chloramphenicol-treated and temperature-shifted *pheS353* cultures. It is interesting to note that Broda (5) produced evidence suggesting that cytoplasmic membrane permeability was altered subsequent to the inhibition of protein synthesis; Davis suggested that streptomycin causes the rapid appearance of membrane damage (8).

**Effects of *rel* genotype.** Strains JP5109 and JP5121 used in the above experiments have "stringent" control of ribonucleic acid synthe-

sis. There is considerable evidence to show, however, that the consequence of amino acid limitation of "relaxed" (*Rel*<sup>-</sup>) strains shows a greater parallelism with the effects of ribosome-inhibiting antibiotics than does limitation of "stringent" (*Rel*<sup>+</sup>) ones (28). We did not attempt to quantitatively compare the morphological changes seen in starved *Rel*<sup>+</sup> and *Rel*<sup>-</sup> cultures, but examined strains carrying each allele for LPS release, outer membrane permeability changes, and spheroplast stability. In all cases where *Rel*<sup>+</sup> cells show altered envelope properties, we find a much more marked effect with *Rel*<sup>-</sup> strains (unpublished data). Other authors found no evidence of envelope changes in starved *Rel*<sup>+</sup> strains under conditions where *Rel*<sup>-</sup> or chloramphenicol-treated cells displayed extensive alterations (19, 22, 30), although one *Rel*<sup>+</sup> strain did show some changes after prolonged starvation (18). Our results are in agreement with other reports in that we find that starved *Rel*<sup>+</sup> strains showed very limited envelope changes at 32 or 37 C, but we did find that extensive envelope alterations occurred during starvation at 42 C (R. R. B. Russell and A. F. Egan, manuscript in preparation).

## DISCUSSION

The SEM data presented here are in substantial agreement with the previous reports of

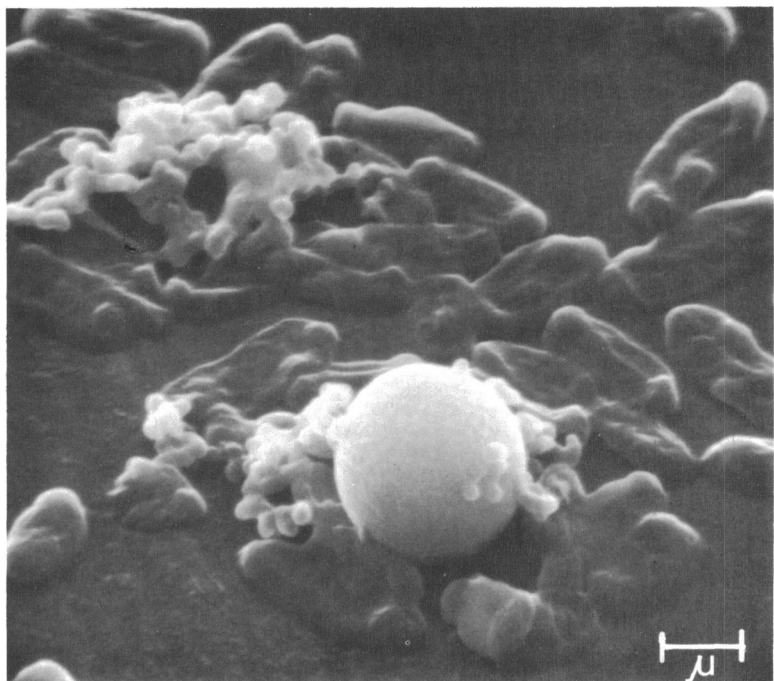


FIG. 5. C1713 treated with 200 μg of streptomycin per ml for 3 h at 42 C.

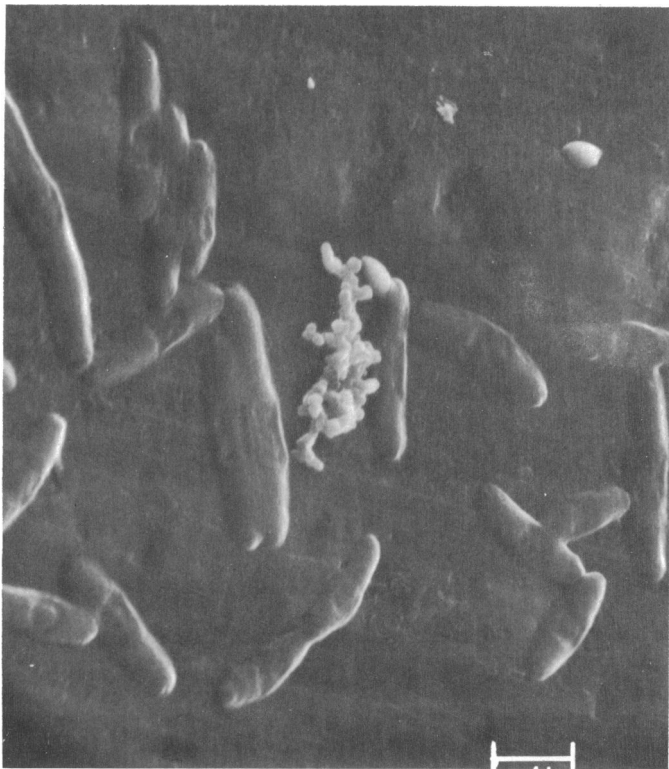


FIG. 6. C1714 (*str<sup>s</sup>*) after 3 h at 42 C.

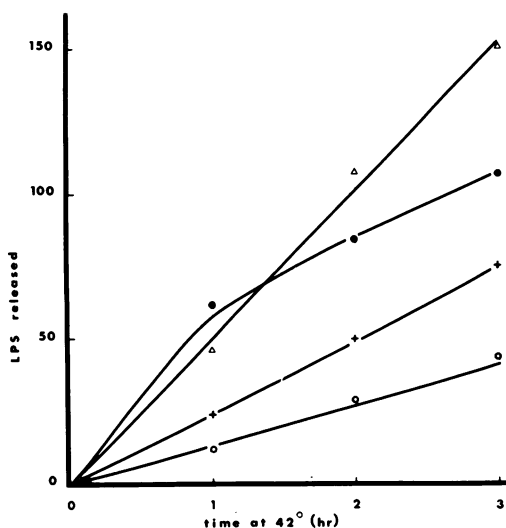


FIG. 7. Release of lipopolysaccharide from cells incubated at 42 C. JP5128, O; JP5128 starved for arginine, ●; JP5128 + chloramphenicol, Δ; JP5121, +. Chloramphenicol (200 μg/ml) was added or arginine removed at zero time. LPS is expressed as counts per minute of [<sup>14</sup>C]galactose in acid-precipitable macromolecule per milliliter of supernatant fluid, divided by the Klett value of the culture at the time of sampling.

TABLE 2. Rifampin susceptibility after incubation at 42 C for 3 h.

Strain	Percent population killed in 20 min by rifampin at:	
	5 μg/ml	100 μg/ml
JP5128	12 (12) <sup>a</sup>	99
JP5128 + CM <sup>b</sup>	73 (81)	90
JP5121 ( <i>PheS</i> <sup>353</sup> )	36 (55)	66

<sup>a</sup> Figures in parenthesis represent the killing by 5 μg/ml when corrected for the reduction in killing rate as indicated by the lethality of 100 μg/ml.

<sup>b</sup> Chloramphenicol (200 μg/ml) was added at time of temperature shift. Results are the mean of at least three experiments.

surface disruption of antibiotic-treated *E. coli* (17). Our study revealed no forms quite like the "raspberries" described earlier (17); the reason for this is unknown, though it may simply be a consequence of different strains being used (*E. coli* K-12 here, and a smooth clinically isolated *E. coli* previously).

The earlier morphological studies (17) did not allow a distinction between the possibility of the antibiotics interfering directly with cell envelope synthesis, or the alternative explanation that the surface changes were only a secondary

consequence of a ribosomal block. It is now clear that preparations of cultures in which protein synthesis has been blocked by amino acid starvation of an amino acid auxotroph, or by inactivation of a temperature-sensitive component of the protein-synthesizing machinery, very closely resemble those of cultures treated with chloramphenicol both in morphology as seen by SEM and in several physiological parameters examined (LPS release, outer membrane permeability, and lysozyme-EDTA susceptibility). Streptomycin induces the same changes as found with the other inhibitory treatments, but in addition appears to cause cellular collapse. The particular morphological appearance of streptomycin-treated cells is, however, also seen with a mutant in which the ribosome is inactivated by mutation, thus demonstrating that all the observed surface manifestations of antibiotic-induced changes are secondary consequences of the ribosomal block rather than due to the antibiotics having secondary sites of action at the membrane.

The mechanism by which the various aberrant forms seen by SEM arise is unclear but would seem in general to be a consequence of unbalanced cellular metabolism, a supposition that is supported by the fact that not all cells respond in the same manner and by the previously reported observation that the development of abnormal forms is extremely dependent

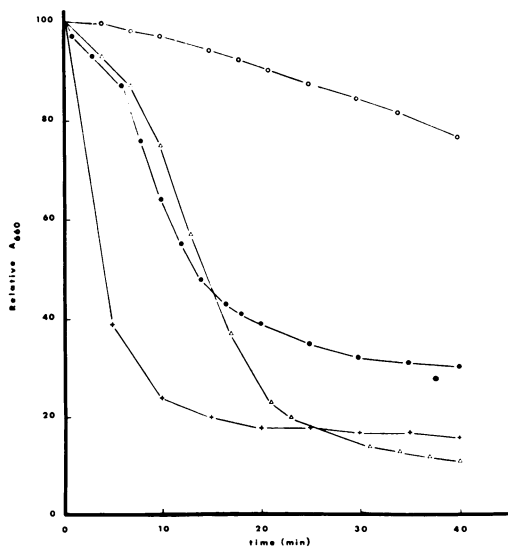


FIG. 8. Lysis after lysozyme-EDTA treatment. JP5128 (O), JP5128 starved for arginine (●) JP5128 + chloramphenicol (Δ), and JP5121 (+) after 3 h of incubation at 42 C with chloramphenicol (200 μg/ml) or starvation as indicated.

on the physiological state of the culture at the time of inhibition, the growth medium used, and the completeness of the inhibition (2, 13, 17, 23).

The mechanism by which outer membrane material (LPS complex) is released from cells and the consequence of the release are not yet elucidated. The outer membrane of gram-negative bacteria acts as a barrier to the penetration of a variety of large molecules, including the antibiotics rifampin and actinomycin D, to their intracellular sites of action (11). In all cases where release of outer membrane complex is observed, we find increased cellular penetrability by rifampin, but it is obviously premature to conclude that the penetrability change is due solely to release of material. A thorough study of both the amount and the composition of the outer membrane material remaining on the cell should throw light on the nature of the barrier.

The clinical significance of the findings reported here can only be considered with caution, but the possibility of synergism between protein inhibitors and other antibacterial factors in the body and the emergence of stable morphological variants is of considerable interest and hopefully will stimulate a re-evaluation of the relationships between antimicrobial therapy and the morphology and role of organisms recovered from clinical material.

Irrespective of whether the above speculations prove of any value, we hope that the results described in this report will contribute to a greater appreciation of the fact that, even though the primary site of molecular action of an antibiotic may be known, the fact that individual members of a population of a particular organism respond in a variety of ways, and show cellular changes not readily predicted from the known site of antibiotic action, may have far-reaching implications for an understanding of its use in chemotherapy.

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